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Lack of Detectable HIV-1–Specific CD8⁺ T Cell Responses in Zambian HIV-1–Exposed Seronegative Partners of HIV-1–Positive Individuals

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Human immunodeficiency virus type 1 (HIV-1)–specific T cell responses were characterized in a blinded study involving infected individuals and their seronegative exposed uninfected (EU) partners from Lusaka, Zambia. HIV-1–specific T cell responses were detected ex vivo in all infected individuals and amplified, on average, 27-fold following in vitro expansion. In contrast, no HIV-1–specific T cell responses were detected in any of the EU partners ex vivo or following in vitro expansion. These data demonstrate that the detection of HIV-1–specific T cell immunity in EU individuals is not universal and that alternative mechanisms may account for protection in these individuals.

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Identifying the correlates of protective human immunodeficiency virus type 1 (HIV-1) immunity is a priority for HIV-1 research and vaccine development. The presence of HIV-1–specific T cell immune responses has been described in HIV-1–exposed but persistently uninfected individuals (EUs) and has been suggested to represent a potential correlate of protection in these individuals, as reviewed in [1]. However, the results from studies aimed at identifying HIV-1– or simian immunodeficiency virus–specific T cell immunity in EUs have varied, and virus-specific CD8⁺ T cell responses were either of low magnitude and breadth, only transiently detectable, not protective, or not readily identifiable to the minimal T cell epitopes targeted in most of the published studies [2–6].

Uninfected partners of HIV-1–infected individuals are at high risk to be exposed to and eventually infected with HIV-1. Cohort studies in HIV-discordant couples have shown that joint testing and counseling prompts a marked increase in reported condom use and an estimated two-thirds reduction in transmission [7]. Nonetheless, residual annual infection rates of 7 cases per 100 couples per year among HIV-discordant couples reflect ongoing exposure [7, 8] and are >14-fold the infection rates among couples with 2 HIV-negative partners [9].

To determine whether HIV-1–specific T cell responses were present in the exposed seronegative partners of adults with chronic HIV-1 clade C infection, we performed a blinded study involving 61 individuals enrolled in Lusaka, Zambia, including 29 EUs.

METHODS

Subjects

A total of 61 individuals enrolled at the Zambia Emory HIV Research Project (ZEHRP) in Lusaka, Zambia, were included in this study. These included 15 low-risk HIV-1–uninfected men and women from 8 concordant HIV-1–negative couples, 14 HIV-1–infected index partners, and 32 partners from 41 discordant couples initially classified as HIV-1 negative. All HIV-positive individuals were antiretroviral therapy naive. High-risk exposure of HIV-1–negative individuals was defined as reporting regular unprotected sex (at least once every 3 months) with an HIV-1–positive spouse, and low-risk exposure was defined as being in a monogamous marriage with an HIV-1–negative partner. Of the 32 partners in HIV-discordant couples initially classified as HIV-1–negative, 3 seroconverted 1–5 months prior to phlebotomy and are classified as HIV-1–positive for the analysis. The remaining 29 individuals were classified as EUs at

the time point of the initial phlebotomy, and 4 of these seroconverted within 1–8 months after the initial phlebotomy. The high rate of HIV-1 seroconversion (7 of 32 individuals within 1 year) reflects the high exposure rate in this cohort. Additional data to estimate exposure were available for 28 of 29 EUs. Among those individuals, 23 couples (82%) reported unprotected sex, 17 (61%) had genital inflammation, 20 (71%) had genital ulceration, 16 (57%) had sperm detected on vaginal swab samples, 5 (18%) had trichomonas detected on vaginal swab samples, and 8 had a pregnancy during the 2-year study period. The respective local institutional review boards approved the study, and all experiments were performed while investigators were blinded for the HIV-1 and exposure status of the participants.

Assessment of HIV-1-Specific T Cell Responses Using Ex Vivo Enzyme-linked Immunosorbent Spot (ELISPOT) Assays

ELISPOT assays were performed using overlapping 13–18 mer peptides (overlap 10aa) spanning the expressed HIV-1-clade C consensus sequence of Gag, Nef, Rev, and Tat [10]. Fresh peripheral blood mononuclear cells (PBMCs) were plated in 96-well plates precoated with anti-interferon (IFN)- γ monoclonal antibody. PBMCs were added at 50,000–100,000 cells/well in 100 μ L of R10 medium (Roswell Park Memorial Institute 1640, 10% fetal calf serum, 10 mM HEPES buffer, 2 mM L-glutamine, and 50 U penicillin-streptomycin per mL). Plates were incubated overnight at 37°C, 5% CO₂, and developed as described [10]. Wells containing PBMCs and R10 were used as negative controls in triplicate. Wells containing PBMCs and phytohemagglutinin served as positive controls. Numbers of spots per well were counted using an automated ELISPOT plate reader, and the number of specific T cells was calculated by subtracting the negative control values. Background was <30/10⁶ PBMCs (3spots/well at 100,000 PBMCs/well) in all cases. Responses were regarded as positive if at least 3 times the mean number of spot forming cells (SFC) in the 3 control wells and >50 SFC/10⁶ PBMCs.

Flow-Based Intracellular Cytokine Staining (ICS)

For 16 individuals, pools of overlapping peptides (OLP) spanning Gag, Nef, and Rev/Tat were used as antigens for flow-based ICS, which was performed directly ex vivo and after 10 days of in vitro stimulation. PBMCs were incubated with peptide pools, anti-CD28, and anti-CD49d antibodies for 1 h at 37°C, 5% CO₂, followed by 5 h in the presence of Brefeldin-A. Cells were stained with surface antibodies (CD3, CD4, CD8; BD) at 4°C for 30 min. After washing, cells were fixed and permeabilized using the Caltag Cell Fixation and Permeabilization kit according to the manufacturer's protocol. ICS was performed using anti-IFN- γ -specific monoclonal antibodies, and cells were analyzed on a FACS Calibur flowcytometry instrument using Fluorescein isothiocyanate, Phycoerythrin, Peridinin chlorophyll

protein, and Allophycocyanin as fluorescent parameters. Control conditions were established using autologous PBMCs, which had not been stimulated with peptide but which had otherwise had been treated identically. PBMCs stimulated with Phorbol-12-myristat-13-acetat/Ionomycin were used as positive controls.

Antigen-Specific In Vitro T Cell Expansion

For antigen-specific in vitro expansion of T cells, 5–10 \times 10⁶ fresh PBMCs were incubated with pools of overlapping peptides spanning Gag, Nef, and Rev/Tat at a final concentration of 2 μ g/mL/peptide. Cell cultures were expanded and maintained in R10 medium supplemented with 50 U/mL rIL-2 for 10 days and then submitted to flow-based ICS as described above.

RESULTS

HIV-1-specific T cell responses from all 61 study participants were analyzed prior to unblinding for HIV-1 infection status. HIV-1-specific T cell responses were initially assessed utilizing fresh PBMCs in an IFN- γ ELISPOT assay with overlapping peptides spanning HIV-1 clade C Gag, Nef, Rev and Tat, as described above [10]. In 2 samples (classified as HIV-1-negative low-risk after unblinding), the initial ELISPOT assays did not pass quality control because of high background activity (>50 SFC/10⁶ PBMCs) in the negative control wells. However, ELISPOT assays in these 2 individuals were repeated from frozen PBMCs and included in the analysis. After unblinding of the HIV-1 status for the 61 subjects (15 low-risk HIV-1-negative individuals, 17 HIV-1-positive individuals, and 29 EUs) strong detectable HIV-1-specific T cell responses directed against Gag, Nef, Rev, and Tat were found in all 17 HIV-1-infected individuals (including the 14 index case patients and the 3 individuals who were infected 1–5 months prior to enrolling into the study), ranging from 700 to 9400 SFC per million PBMCs (median value, 1700 SFC per million PBMCs; Figures 1A and 1C). In these infected individuals, HIV-1-specific T cell responses were preferentially directed against Gag and Nef (Figure 1C), in concordance with previous studies [10]. No HIV-1-specific T cell responses were detected in the 15 HIV-1-negative, low-risk individuals. Among the 29 high-risk EU subjects, HIV-1-specific T cell responses were detected in only 1 individual (AD45; Figure 1A). This study participant was classified as HIV-1 seronegative during the initial blood draw on the basis of a serological p24 enzyme-linked immunosorbent assay (ELISA) result that was negative for HIV 1 month prior to phlebotomy. However, the patient was in fact identified and assayed during acute HIV-1 infection with subsequent HIV-1 seroconversion (the patient's first positive HIV ELISA result was obtained 7 weeks after phlebotomy). Primary HIV-1 infection at time of the T cell assays was documented by outgrowth of replication

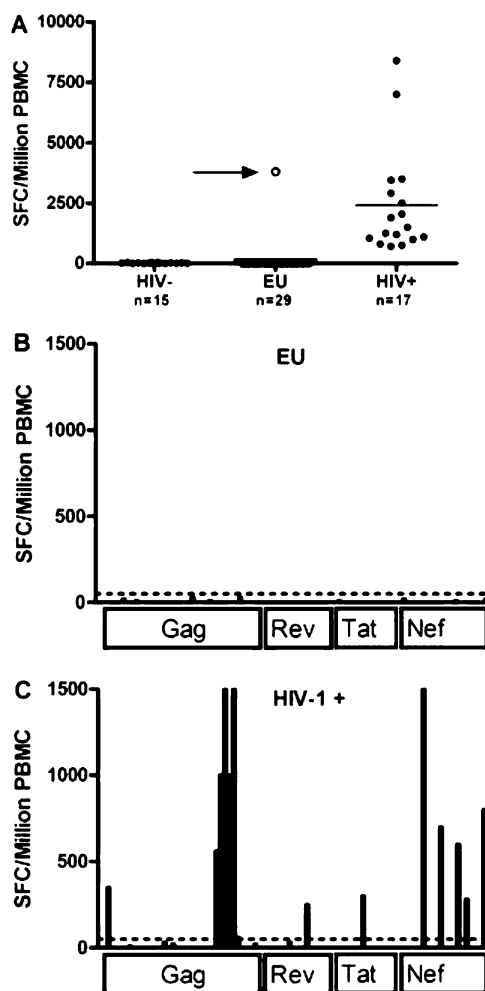


Figure 1. Human immunodeficiency virus type 1 (HIV-1)-specific T cell responses were measured in blinded fashion by ex vivo interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay in HIV-1 seropositive and exposed seronegative individuals. ELISPOT data were available for all 61 study subjects. Panel A shows strong T cell responses to HIV-1 clade C Gag, Rev, Tat, and Nef overlapping peptides in all individuals identified as known HIV-seropositive study participants after unblinding. Results are shown as total magnitude of SFC/ 10^6 peripheral blood mononuclear cells (PBMCs). In contrast, study subjects who were identified as HIV-1 seronegative at study entry (including 28 high-risk exposed uninfected [EU] and 15 low-risk HIV seronegative control subjects) did not have detectable HIV-1 clade C-specific T cell responses ex vivo. One individual (AD45; arrow) who was classified as EU at study entry by an enzyme-linked immunosorbent assay (ELISA) result that was negative for HIV at 1 month prior to phlebotomy had detectable T cell responses by ex vivo ELISPOT. This individual had a positive HIV ELISA result at 7 weeks after phlebotomy and was sampled and assayed during acute HIV infection (confirmed by isolation of replication-competent virus from a PBMC sample from same time point as the ELISPOT assay; no plasma was available for HIV viral load quantification). Panels B and C show representative examples of ELISPOT screenings with overlapping peptides spanning HIV-1 clade C Gag, Rev, Tat, and Nef in an HIV-1-seropositive individual and a high-risk exposed seronegative individual, respectively. All experiments were performed blinded. Positive responses were defined as at least 3 times the number of SFCs in the control wells and had to be >50 SFC/ 10^6 PBMCs [10].

competent virus from stored PBMCs from this time point; however, unfortunately, no plasma was available to quantify the actual HIV viral load. No HIV-1-specific T cell responses were detected in any of the remaining 28 HIV-1-seronegative high-risk EUs (Figures 1A and 1B), including 1 additional individual with HIV-1 seroconversion within 2 months after phlebotomy. Taken together, these data demonstrate the absence of detectable ex vivo HIV-1 Gag-, HIV-1 Nef-, HIV-1 Rev-, and HIV-1 Tat-specific T cell responses in exposed, uninfected partners of HIV-1-infected individuals using an IFN- γ ELISPOT assay that had 100% sensitivity to detect virus-specific T cell responses in individuals with chronic HIV-1 infection in this study, as well as in the 1 individual with acute HIV-1 infection, when cytotoxic T lymphocyte responses are generally of low magnitude and breadth [11].

Ex vivo frequencies of HIV-1-specific T cell responses can be of low magnitude in EUs, as described in previous studies [1, 5]. We therefore used an in vitro expansion protocol on fresh samples obtained from a subset of 16 study subjects (4 EUs, 4 low-risk HIV-1-negative individuals, and 8 HIV-1-infected individuals) during a second blood draw 12 months later, prior to unblinding, to expand HIV-1-specific T cells, using Gag, Nef, Rev, and Tat pools of overlapping peptides as described above. In the 8 HIV-1-infected individuals studied, HIV-1-specific CD8 $^{+}$ T cell responses were detectable in all individuals directly ex vivo, with the total response ranging from 0.34% to 4.06% of CD8 $^{+}$ T cells (median value, 1% of CD8 $^{+}$ T cells) (Figures 2A and 2E). Ex vivo frequency of CD8 $^{+}$ T cells directed against Gag ranged from 0.4% to 3.5%, Nef-specific CD8 $^{+}$ T cells ranged from 0.3% to 2.7%, and CD8 $^{+}$ T cells directed against Rev/Tat ranged from 0.2% to 1.5%. These virus-specific CD8 $^{+}$ T cell responses were expanded significantly following in vitro stimulation (median increase, 27-fold; range, 9–69-fold; $P = .002$), reaching median frequencies of 33% of total CD8 $^{+}$ T cells (range, 6%–80%) (Figures 2B and 2E). Despite this significant expansion of HIV-1-specific CD8 $^{+}$ T cells in vitro in infected individuals, no HIV-1-specific CD8 $^{+}$ T cell responses above background were detected in the 8 HIV-1-negative individuals (4 low-risk and 4 high-risk seronegative subjects) either when tested directly ex vivo or following the in vitro expansion (Figures 2C, 2D, and 2E). The 4 high-risk seronegative individuals reported condom use and exposure histories similar to those reported by the overall group of 28 EUs. Three of the 4 couples reported unprotected sex, 1 of 4 EUs had genital inflammation, 2 of 4 EUs had genital ulceration, and 2 of 4 EUs had sperm detected on vaginal swab samples.

DISCUSSION

This study, which was performed entirely blinded with pre-established criteria for assay positivity, demonstrates a lack of

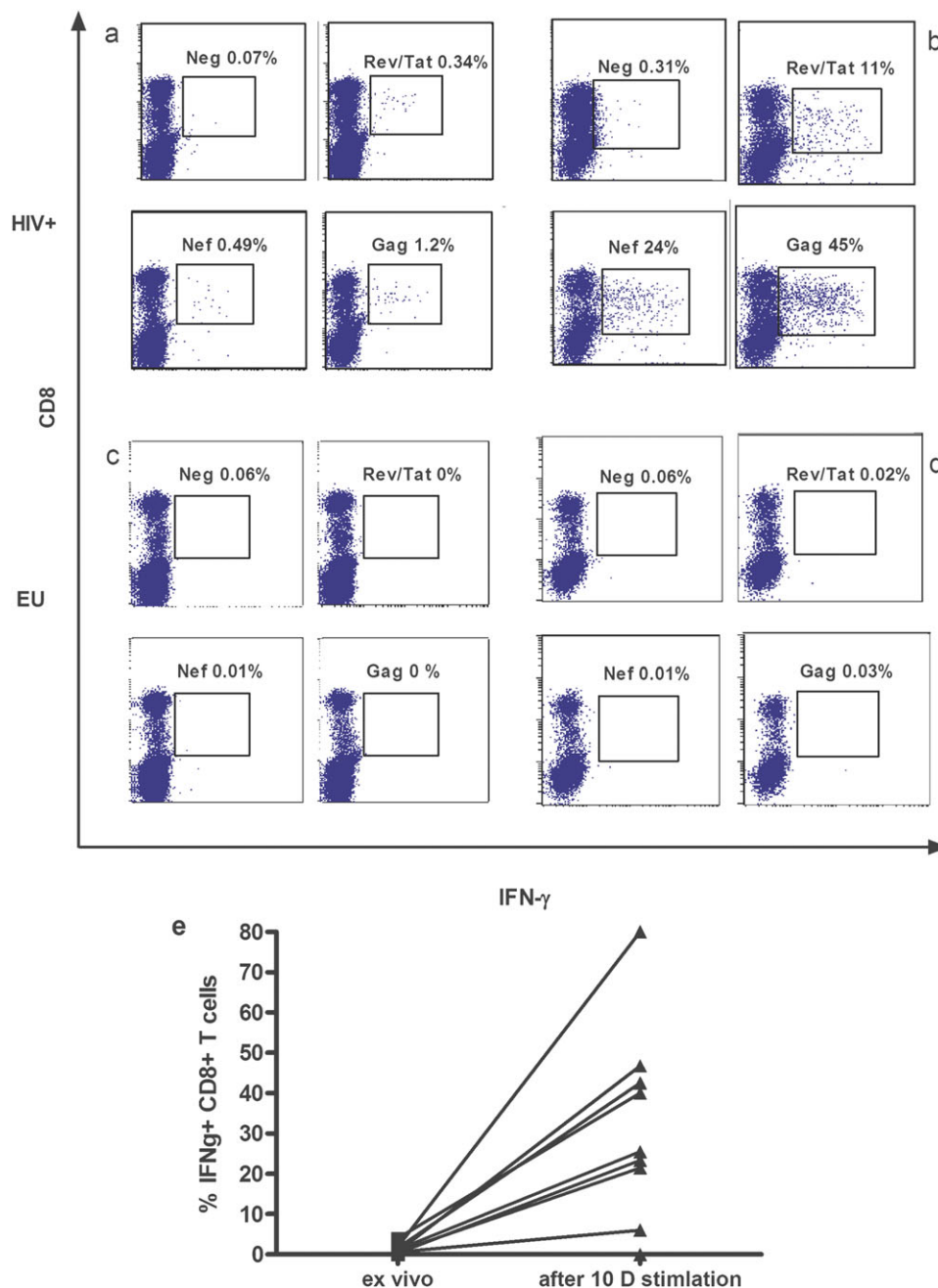


Figure 2. Large expansion of human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cell responses measured by flow cytometry in HIV-1-positive (but not HIV-1 high- or low-risk seronegative) individuals after HIV-1-specific in vitro stimulation and expansion. HIV-1 clade C Gag, Rev, Tat, and Nef responses were measured by flow cytometry ex vivo and after 10-day in vitro peptide-specific stimulation with pools of overlapping peptides in a subset of HIV-positive ($n = 8$) and HIV-seronegative individuals ($n = 8$), including 4 high-risk and 4 low-risk EU. Representative examples of the total HIV-1-specific intracellular interferon- γ response ex vivo (A and C) and after 10-day stimulation (B and D) for an HIV-1-positive individual (A and B) and an exposed HIV-1-uninfected individual (EU) (C and D) are shown. Panel E shows the summary for the total HIV-1-specific T cell responses for 16 individuals tested pre- and post-stimulation as measured by flow cytometry. HIV-1-specific CD8+ T cell responses were detectable in all HIV-1-infected individuals directly ex vivo, with the total response ranging from 0.34% to 4.06% (median response, 1%). These virus-specific CD8+ T cell responses expanded significantly after in vitro stimulation (median increase, 27-fold; range, 9–69-fold; $P = .002$), reaching median frequencies of total HIV-1-specific CD8+ T cells of 33% (range, 6%–80%). No HIV-1-specific T cell responses above background were detected by intracellular cytokine staining in the 8 HIV-1-negative individuals (4 low-risk and 4 high-risk seronegative subjects) who were tested directly ex vivo or following the in vitro expansion (C, D, and E), as represented by the graph line overlying the x-axis, showing no change between before and after stimulation (E).

detectable HIV-1-specific T cell responses in a group of EU partners of HIV-1-infected individuals in Lusaka, Zambia. Several mechanisms might account for the lack of detectable virus-specific T cell responses. Sexual exposure to HIV-1 is difficult to quantify and validate, and exposure in our study subjects might have been low. However, epidemiological data in this cohort strongly suggest ongoing regular HIV-1 exposure [7, 8], and condom use, exposure frequencies, and genital examination findings, as well as the acquisition of HIV-1 in 7 of 32 high-risk individuals enrolled within months of phlebotomy, confirm this finding. Despite counseling for condom use, HIV-1 incidence rates in this cohort of HIV-discordant couples have remained high (7%–8% per year), as have the rates of pregnancies (22% per year) [12]. Overall, these data strongly suggest high HIV-1 exposure rates in the HIV-1-negative partners.

An alternative explanation for the lack of detectable HIV-1-specific T cell responses can be low sensitivity of the assays used to detect low-frequency HIV-1-specific T cell responses. Previous studies have used *ex vivo* ELISPOT and flow cytometry assays to detect immune responses in EU, and using these same assays, we detected HIV-1-specific T cell responses in all HIV-1-infected individuals, including an individual with primary HIV-1 infection, when HIV-1-specific T cell immunity is generally low [11]. We furthermore enhanced the sensitivity of the assays used in this study by a specific *in vitro* expansion of HIV-1-specific T cells following stimulation with HIV-1 antigens. This *in vitro* expansion resulted in a median 27-fold increase in HIV-1-specific T cell responses in infected individuals but no detectable T cell responses in EUs. However, we cannot exclude the possibility that differences in the experimental approaches used here and in previously published studies [13] might have accounted for some of the divergent results reported in the literature in terms of presence or absence of HIV-1-specific T cell responses in EUs. However, overall, these data suggest that the absence of detectable responses was not a consequence of lack of exposure or of the sensitivity of the assays used.

In conclusion, we here report the absence of detectable HIV-1-specific T cell responses in a cohort of HIV-1-exposed but persistently uninfected partners of HIV-1-infected individuals from a well-established and well-characterized Zambian cohort. These data demonstrate that the detection of virus-specific immunity in EUs is not universal, as suggested by an increasing body of data [5, 6], and that other mechanisms than HIV-1-specific T cell immunity might account for the protection of EU individuals [14, 15]. Additional blinded prospective studies in large cohorts of EUs, including studies that involve sampling at mucosal sites of exposure, are needed

to systematically identify the correlates of protection from infection in these individuals.

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